SEASONAL AND HORMONE INDUCED CHANGES IN THE TESTES OF *RANA PIPIENS*

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The general conclusions of the majority of those biologists who have experimented with the frog *Rana pipiens pipiens* (Schreber) indicate that the male frog is an excellent test animal for the early diagnosis of human pregnancy. Nevertheless there are some unanswered questions which affect the use of this test. One of these is the possibility of seasonal variation in sensitivity in the frog, a period of time when the test animal is less responsive to chorionic gonadotrophin than in other seasons. This period of lessened sensitivity occurs during the summer months. It is important to determine the reason for this seasonal sensitivity since during this time false negative results for pregnancy tests are most frequently reported.

A histological study has been made of seasonal differences in the maturation and release of spermatozoa by *R. pipiens* after injections of concentrated pregnancy urine and injections of fresh anterior lobes of frog pituitary glands. The results are compared with the seasonal picture of testes of treated frogs. A study of the condition of the testes during the various seasons of the year has helped to answer the question of why there is seasonal variation in sensitivity of this species.

The purposes of this study were (a) to determine the variations in seasonal sensitivity of the male frog, *R. pipiens*, to 2.5 cc injections of pregnancy urine concentrate, (b) to compare the testes of frogs injected with pregnancy urine with testes of uninjected frogs, (c) to compare the testes of both pregnancy urine injected and uninjected frogs with frogs that have been injected with fresh anterior lobes of pituitary glands, and (d) to determine if the use of pregnancy urine concentrate during the summer months might be used as a safeguard against false negative results. To many using the test in the past, seasonal variation in sensitivity has appeared as quantitative variations in the number of mature spermatozoa per microscope field. The basis for this should be apparent in a histological examination of the testes, demonstrating the location and number of mature spermatozoa.

In 1948, Wiltberger and Miller first presented the use of *R. pipiens* as a new test animal for diagnosis of early pregnancy and suggested the possibility of seasonal differences in the reactions of frogs. Most investigators, with the exception of Cutler (1949) and Marsters, Black, and Randall (1950) report the male frog *R. pipiens* to be less sensitive during the summer months as indicated by quantitative estimates. Therefore, during August, when sensitivity of this species appears to be the lowest, another species, *Rana clamitans*, was compared with it in respect to the condition of the testes to determine if *R. clamitans* may be employed during August. This is the breeding season for *R. clamitans* in northern states. It also was necessary to determine whether cyclic spermatogenesis is the sole cause of reduced reactiveness in summer, as compared to other seasons of the year.

EXPERIMENTAL PROCEDURE

First morning urine specimens of a woman in her first trimester of pregnancy were collected daily during March, April and May, and stored in pint jars in a refrigerator at 10° C. These urine specimens were used in all of the experiments which were performed during that year. Apparently, the chorionic gonadotrophic hormone of pregnancy urine retains its potency for at least a year *if kept in a refrigerator at 10° C.* This observation indicates that the testing of a urine sample

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immediately after voiding is not necessary for accurate clinical results. However, before each experiment was performed the urine was checked for potency on a test frog.

Male *R. pipiens* weighing 30 to 35 gm were received from Vermont on the first day of every month. Upon arrival they were placed in a refrigerator at 10° C in about one and one-half inches of water. The frogs were held in the refrigerator for four or five days before being used. A testis was removed from a normal uninjected frog and fixed for sectioning to be used as a check.

The frog to be operated on was etherized lightly and placed on a clean towel. A small incision was made through the skin on the left ventral abdominal surface, at the border of the spotted area and anterior to the hip. The muscle of the body wall was pierced and torn with tweezers to the size of the slit made in the skin. The fat bodies and the left testis were located. The mesorchium was cut and the left testis removed and placed in Bouin's solution for fixation. In handling the testis was held by the mesorchium so that it would not be mutilated in any way, since, according to Rugh (1939) manipulation of the testis may force the release of some spermatozoa into the seminiferous tubules. This was considered a normal unstimulated testis. It is believed that the testis removed from an experimental animal before injection of the hormone serves as a better control than the testis from a different animal. With fine silk thread and a small surgical needle the muscle layer of the body wall and skin layer were closed. The frog was then placed in a one-quart jar at the bottom of which there was sterile cotton moistened with Ringer's solution.

After approximately one and one-half hours, when the operated frog had recovered activity, 2.5 cc of pregnancy urine concentrate were injected into the dorsal lymph sac. The frog remained in the jar at room temperature, occasionally being moistened with small amounts of Ringer's solution. After three hours the frog was again etherized and the right testis removed and fixed. To check for possible individual variation, in each case three frogs were operated on in this manner and the results compared.

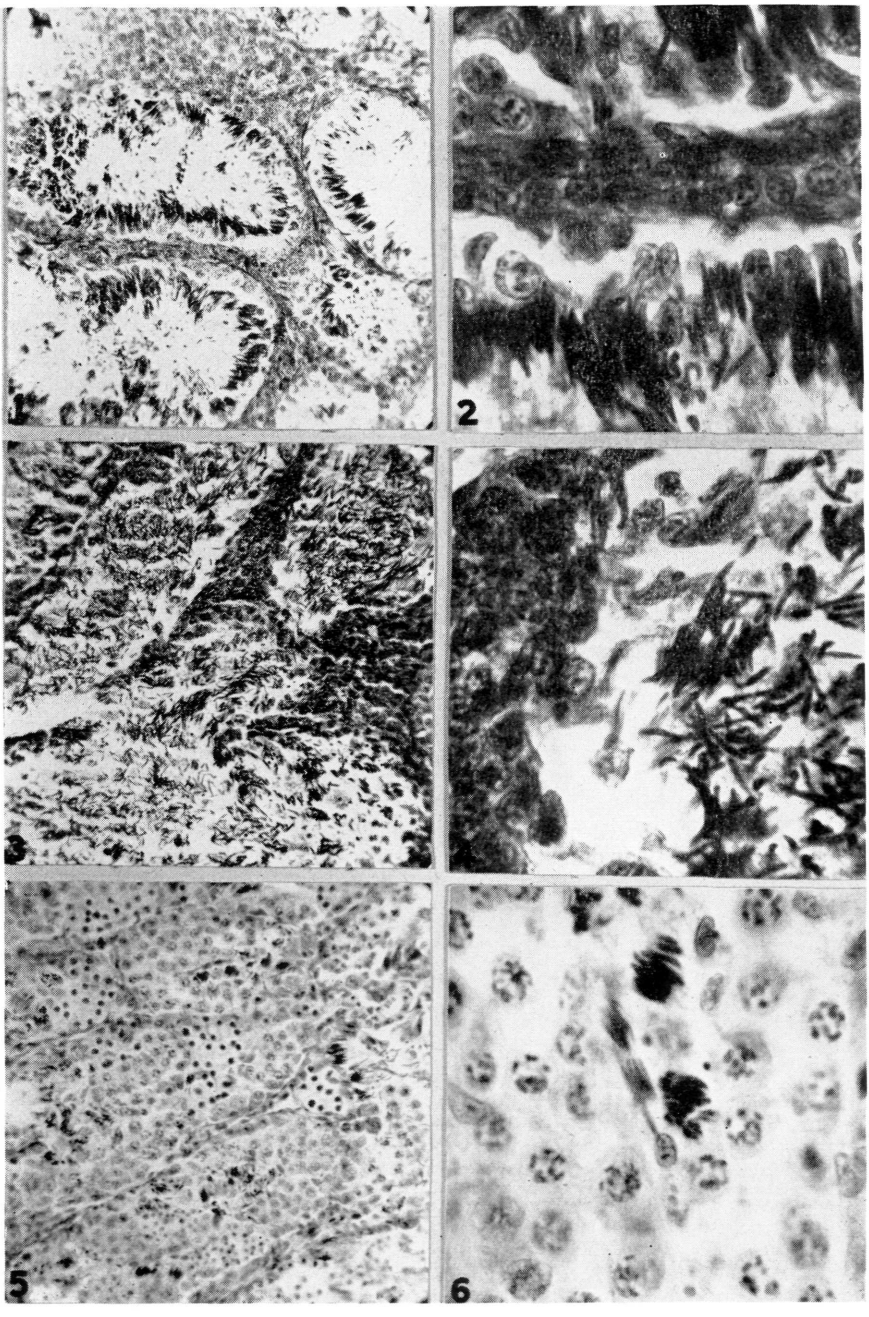
In order to determine how early the effect of the gonadotrophins could be detected in sections of testes, two sets of experimental animals were run and their testes removed at one-half hour, one hour and two hours.

To compare the effects of chorionic gonadotrophins with gonadtrophins of the pituitary four anterior lobes of male *R. pipiens* pituitary glands were injected into the dorsal lymph sac of frogs during each month and the results compared.

EXPLANATION OF FIGURES IN PLATE I

1. Sectioned testis of the uninjected frog in March. Several seminiferous tubules with spermatozoa clustered at the periphery.
2. Sectioned testis of the uninjected frog in March. A portion of two tubules of #1 under oil immersion.
3. Sectioned testis of the frog in March after injection of pregnancy urine concentrate. Several tubules with spermatozoa scattered freely in the lumen.
4. Sectioned testis of the frog in March after injection of pregnancy urine concentrate. A portion of #3 under oil immersion.
5. Sectioned testis of the uninjected frog in August. Lumen of the seminiferous tubules completely filled with maturation stages, and spermatozoa in clusters.
6. Sectioned testis of the uninjected frog in August. An enlarged portion of one seminiferous tubule with maturation stages and clumped spermatozoa.

All material illustrated was prepared from *Rana pipiens*, except where indicated, and was fixed in alcoholic Bouin's picro-formol solution, sectioned at 10 microns, and stained with Delafield's hematoxylin and eosin.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RESULTS

Condition of testes prior to the breeding season. In March, the sectioned testes of uninjected frogs show spermatozoa in clusters outlining the periphery of the seminiferous tubules (figs. 1, 2). Spermatogonial cells, primary and secondary spermatocytes, and spermatid cysts are rare. The germinal layers are either absent or one or two layers may be present, along with spermatogonial cells in some tubules.

The histological picture of the testes of frogs in March after an injection of known pregnancy urine concentrate shows spermatozoa within the lumen of the seminiferous tubules (figs. 3, 4). The spermatozoa are scattered freely, and are not found in groups or clusters as in the sections of uninjected frog testes. There is a definite disturbance in the testicular picture of frogs that had been injected with pregnancy urine.

Condition of testes during the breeding season. April and May are the months when *R. pipiens* normally breeds in northern states. The breeding season varies depending upon the latitude and upon variations in the temperature. The histological picture of the testis of one uninjected frog in April, for example, shows nearly all spermatozoa on the periphery of the tubules. The spermatozoa are found closely clustered into groups. Stages of spermatogenesis are rare and therefore there are only one or two germinal layers present.

After stimulating the frog by means of an injection of pregnancy urine concentrate during the breeding season, three-fourths of the spermatozoa are released and discharged into the lumen. Some are clumped together into groups which are found in the lumen. Such clumps are often seen in the frogs urine during a pregnancy test. Not all tubules are affected equally. There may be some tubules with no scattered spermatozoa. In these cases the spermatozoa are clustered at the periphery of the tubules.

Condition of testes after the breeding season. The sectional testes of uninjected frogs in June and July show some spermatozoa still present which are located at the periphery of the tubules. Frogs injected with pregnancy urine concentrate in June and July show a picture similar to those frogs that were injected during March, April, and May with the exception that fewer spermatozoa are present. In August very few spermatozoa are found (figs. 5, 6). The tubule is almost completely filled with maturation stages, especially with numerous early prophase 1 stages. In August there are four to eight germinal layers in comparison to the two

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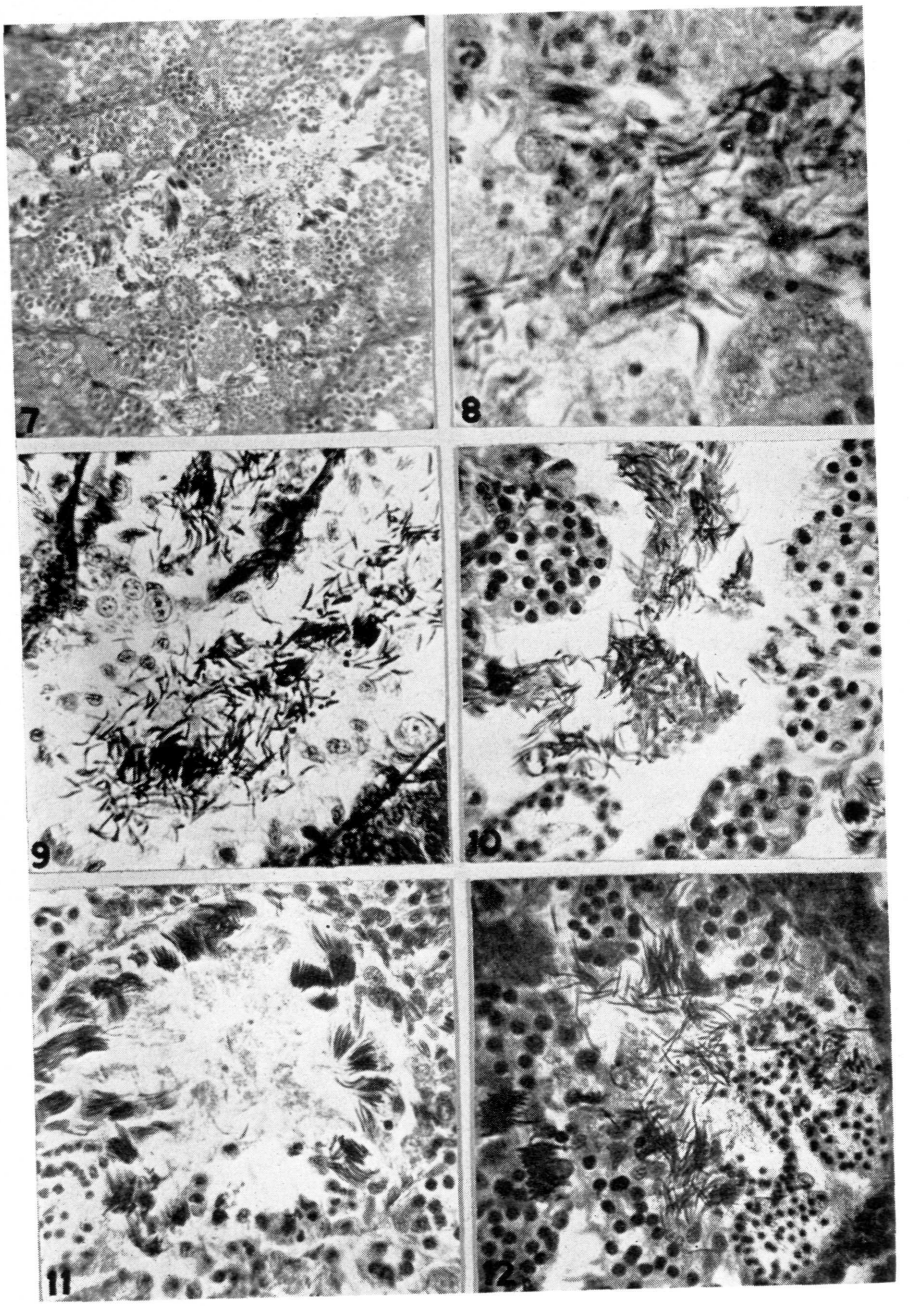
EXPLANATION OF FIGURES IN PLATE II

7. Sectioned testis of the frog in August after injection of pregnancy urine concentrate. Several tubules with the lumen filled with maturation stages; scattered spermatozoa, with some still grouped.
8. Sectioned testis of the frog in August after injection of pregnancy urine concentrate. An enlarged portion of a seminiferous tubule with stages of maturation, and scattered spermatozoa.
9. Sectioned testis of the frog in March after injection of four anterior lobes of frog pituitary glands. Spermatozoa are scattered freely in the lumen.
10. Sectioned testis of *R. clamitans* in August after injection of four anterior lobes of frog pituitary glands. Spermatozoa are scattered in the lumen.
11. Sectioned testis of uninjected *R. clamitans* in August. Spermatozoa are clustered near the periphery of the tubules.
12. Sectioned testis of *R. clamitans* in August after injection of pregnancy urine concentrate. Abundant spermatozoa and spermatid stages.

All material illustrated was prepared from *Rana pipiens*, except where indicated, and was fixed in alcoholic Bouin's picro-formol solution, sectioned at 10 microns, and stained with Delafield hematoxylin and eosin.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or three germinal layers in April and May. After injections in August a smaller number of spermatozoa were found as compared to other months. Practically the entire tubule was filled with cells undergoing maturation divisions (figs. 7, 8).

Condition of testes before the hibernation season. September and October can be considered the pre-hibernating period in *R. pipiens*. Sections of testes of uninjected frogs in October differ from those in August in that there are more spermatozoa present, more late maturation stages are present, and there is a noticeable increase in the size of the lumen of the tubules. The testes sectioned after injections of pregnancy urine concentrate show results similar to those described for March.

Condition of testes during the hibernation season. The histological picture of the testes of uninjected hibernating frogs during November, December, January, and February appears similar to the testes of uninjected frogs in March. The spermatozoa are abundant during the frogs hibernation period and are attached to the border of the seminiferous tubules. There are one to three germinal layers present including secondary spermatocyte and spermatid stages. Frogs injected with pregnancy urine concentrate during these months show the characteristic testicular picture.

During November and February, testes had been removed from frogs after intervals of one-half hour, one hour, and two hours following the injection of pregnancy urine concentrate. In all cases the left testis of the uninjected frog was compared to the right testis of the same frog after an injection of pregnancy urine concentrate. In all cases there was an apparent stimulation.

Collectively these results are consistent in that all the frogs stimulated either with pregnancy urine concentrate or anterior lobes of pituitary glands (figs. 9, 10) demonstrated the pronounced effect of the release of spermatozoa into the seminiferous tubules.

In August the sectioned testis of uninjected *R. pipiens* was compared with the sectioned testis of uninjected *R. clamitans* (fig. 11). It is during this month that the responsiveness of *R. pipiens* to injection of pregnancy urine appear to be most depressed. In sectioned testes of *R. clamitans* the spermatozoa are clustered and grouped together at the periphery of the tubules. There are some maturation stages and many tubules have an abundance of spermatid stages. In sectioned testes of *R. pipiens* there are fewer spermatozoa and maturation stages almost completely close the lumen of the tubules. After injections of pregnancy urine concentrate the sectioned testes of *R. clamitans* (fig. 12) shows abundant release of spermatozoa.

From the histological evidence it seems apparent that during the summer months cyclic spermatogenesis is the cause of lessened reactiveness of the male frog, *R. pipiens*, to injections of pregnancy urine concentrate. It also appears that in *R. clamitans* more spermatozoa are present and therefore there is a greater number of spermatozoa released after the injections.

Photomicrographs of sectioned testes after pregnancy urine injections and after injections of fresh anterior lobes of pituitary glands during all seasons of the year can be found in a dissertation by Biesinger (1951) in The Ohio State University library.

CONCLUSIONS

1. A study of the sectioned testes of the uninjected male frog, *R. pipiens*, discloses cyclic variations, indicating seasonal changes. The histological appearance of the testes of the uninjected frog prior to the breeding season in March (figs. 1, 2) and during the breeding season (April, May) shows spermatozoa in clusters and closely grouped together at the periphery of the seminiferous tubules.

2. The sectioned testes of frogs injected with pregnancy urine concentrate show a definite testicular change since after the injections there is a release of spermatozoa into the lumen of the tubules (figs. 3, 4, 7, 8, 12).

3. The effects of injections of pregnancy urine concentrate are similar to the effects of injections of anterior lobes of pituitary glands (figs. 9, 10).

4. From the histological results it seems apparent that during the summer months, cyclic spermatogenesis is the cause of reduced sensitivity of the male frog, *R. pipiens* to injections of pregnancy urine concentrate (figs. 5, 6). This lessened activity suggests the advisability of using concentrate of pregnancy urine for pregnancy tests as a safeguard against false negative results, especially during the post-breeding season.

5. During August, the sectioned testes of uninjected *R. clamitans* (fig. 11) show a larger number of spermatozoa attached to the basement membrane of the tubules than found in *R. pipiens* (figs. 5, 6). It may be suggested that *R. clamitans*, if it is available, would make a satisfactory substitute for *R. pipiens* during this month.

6. There is no apparent difference in the testicular picture due to time lapse after the pregnancy urine injections. Frogs allowed to stand for one-half hour, one hour, two hours, and three hours were not essentially different.

7. The chorionic gonadotrophic hormone of pregnancy urine retains its potency for at least one year if stored in a refrigerator at 10° C. This observation indicates that the testing of a urine sample soon after voiding is not necessary for accurate clinical diagnosis.

8. Since the chorionic gonadotrophic hormone does not seem to deteriorate readily, samples of pregnancy urine may be stored and used for demonstrations, as for producing live spermatozoa for classroom purposes.

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